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Citation for published version:

Donaldson, K, Brown, DM, Mitchell, C, Dineva, M, Beswick, PH, Gilmour, P & Macnee, W 1997, 'Free radical activity of PM10: iron-mediated generation of hydroxyl radicals', *Environmental Health Perspectives*, vol. 105, no. Suppl 5, pp. 1285-9. <<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1470141/>>

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Environmental Health Perspectives

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Free Radical Activity of PM₁₀: Iron-mediated Generation of Hydroxyl Radicals

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The purpose of this study was to test the hypothesis that particulate matter $\leq 10 \mu\text{m}$ in aerodynamic diameter (PM₁₀) particles have the ability to generate free radical activity at their surface. We collected PM₁₀ filters from the Edinburgh, United Kingdom, Enhanced Urban Network sampling site, removed particles from the filter, and tested their ability to cause free radical damage to supercoiled plasmid DNA. We found that the PM₁₀ particles did cause damage to the DNA that was mediated by hydroxyl radicals, as shown by inhibition of the injury with mannitol. The PM₁₀-associated hydroxyl radical activity was confirmed using a high-performance liquid chromatography-based assay to measure the hydroxyl radical adduct of salicylic acid. Desferrioxamine abolished the hydroxyl radical-mediated injury, which suggests that iron was involved. Analysis of PM₁₀ filters confirmed the presence of large amounts of iron and leaching studies confirmed that the PM₁₀ samples could release substantial amounts of Fe(III) and lesser amounts of Fe(II). To investigate the size of the particles involved in the hydroxyl radical injury, we centrifuged the suspension of PM₁₀ to clarify, tested the clear supernatant, and found that it had all of the suspension activity. We conclude, therefore, that the free radical activity is derived either from a fraction that is not centrifugeable on a bench centrifuge, or that the radical generating system is released into solution. — *Environ Health Perspect* 105(Suppl 5):1285–1289 (1997)

Key words: PM₁₀, air pollution, free radical, oxidative stress, environmental lung disease

Introduction

Recent research has confirmed the adverse health impacts of particulate air pollution in causing acute mortality, impairment of lung function, and exacerbation of airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma (1,2). PM₁₀ is the commonly used measure of respirable environmental suspended particulate material (SPM) and represents airborne particles less than 10 μm aerodynamic diameter collected with 50% efficiency; smaller particles are also collected with variable efficiency (3). In the United Kingdom, a standard of 50 $\mu\text{g}/\text{m}^3$ PM₁₀ was recently

recommended by a Department of the Environment committee (3). The average PM₁₀ concentration for British cities ranges between 10 and 50 $\mu\text{g}/\text{m}^3$, with short-term peaks extending to 100 $\mu\text{g}/\text{m}^3$ (3,4), but in other countries much higher levels can be experienced.

Little data are available on the detailed composition of PM₁₀ and the composition is highly variable between locations (3,4). The association between mortality and pulmonary symptoms with SPM in diverse geographic locations such as New York, London, Utah, and Philadelphia,

Pennsylvania, where the sources ranged from predominantly coal combustion to automobile emissions to a steel mill (5), suggests that the detailed composition of the SPM is not critical but that the total mass of fine particles is important. PM₁₀ particles can be in very fine size ranges (6). Particles in the 20 nm size range are common, and represent relatively small masses but large particle numbers (7).

It has been hypothesized that direct interactions between deposited fine particles and the pulmonary epithelium in the terminal bronchiolar/proximal alveolar regions is critical for harmful effects of PM₁₀ particles (8). We extended the hypothesis to suggest that PM₁₀, particularly an ultrafine component of it, together with other ultrafine materials, may have free radical activity that contributes to its toxicity. In this paper we suggest that, as in findings with other particles, free radicals may arise from Fenton chemistry involving iron present on the particles (9–11). Additionally, we investigated whether iron could be leached from PM₁₀ particles to a greater degree at the acid pH found in the phagolysosome (12).

Materials and Methods

Preparation of PM₁₀

The PM₁₀ was provided on filters taken directly from the tapered element oscillating microbalance in U.K. Enhanced Urban Network monitoring site stationed in Princes Street Gardens in Edinburgh. The filters were supplied by J. Hunter, Edinburgh District Council, Department of Environmental Services. Filters were quartered, and each quarter was placed in 100 μl sterile H₂O, 4 mM mannitol (BDH Chemicals, Poole, U.K.) or 4mM desferrioxamine-B (DSF-B) (Sigma Chemicals, Poole, U.K.) solution as required. Each sample was sonicated in a sonicating water bath for 30 sec and vortexed vigorously to remove PM₁₀ from the filter into solution. Blank filters without PM₁₀ were similarly treated for control purposes. The mean \pm SEM weight of particulate matter on the filters was $996.2 \pm 181.8 \mu\text{g}/\text{filter}$. Although it is impossible to quantify the weight of particulates removed by sonication, we estimated by comparative turbidometry against carbon black standards that with our standard procedures, 10 to 30% of the particulate matter was removed from the filters into solution. In some

This paper is based on a presentation at The Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at EHP 27 March 1997; accepted 7 July 1997.

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Abbreviations used: COPD, chronic obstructive pulmonary disease; 2,3 DHBA, 2,3 dihydroxybenzoic acid; DSF-B, desferrioxamine-B; EDXA, energy dispersive X-ray analyzer; HPLC, high-performance liquid chromatography; PM₁₀, particulate matter $\leq 10 \mu\text{m}$ in aerodynamic diameter; SPM, suspended particulate matter.

experiments, PM₁₀ samples prepared in either H₂O or DSF-B were centrifuged for 10 min at 13,000 rpm to remove large insoluble particles from the solution. The resulting clear supernatant was added to the DNA assay at the same concentrations as the uncentrifuged PM₁₀ solutions.

Iron Release from Particles

Iron release was measured under the two pH conditions of pH 4.6 and 7.2. PM₁₀ samples were suspended in either pH 4.6 or 7.2 citric acid/NaHPO₂ (BDH Chemicals) buffer as described above. Following an 8-hr incubation at 37°C in a rotating incubator, larger visible particles were removed by centrifugation at 2500 rpm for 10 min. By our estimation, each quarter filter should provide a maximum of 75 µg PM₁₀ particles. Iron concentration released after 8 hr was determined by the addition of either 4 mM DSF-B (Sigma Chemicals) for Fe(III) determination, or 4 mM ferrozine (Sigma Chemicals) for Fe(II) determination. Quantification of the particle-leached Fe(II) or Fe(III) was measured by spectrophotometry at 562 and 430 nm, respectively, and comparison with Fe(II) or Fe(III) standards of FeSO₄ and FeCl₃ (10). The limit of sensitivity of this assay was 0.5 nmol. The amount of iron leached from 74.7 µg of particles after an 8-hr incubation was expressed as nanomol Fe(II) or Fe(III).

Free Radical Activity of PM₁₀

Plasmid Assay. Two hundred and ninety nanograms ϕ X174 RF1 DNA (Gibco Europe, Paisley, U.K.) was incubated with PM₁₀ particles or PM₁₀ particles treated as described above (10). Briefly, all DNA control and treatment samples were incubated for 8 hr at 37°C prior to agarose gel electrophoretic separation of the supercoiled, relaxed coil, and linear fragments of the DNA. The amount of supercoiled DNA present after treatment, compared to control, indicated the level of free radical-mediated damage to the plasmid. From our estimation of particles removed from each filter, the weight of particles in 5 µl should be 3.7 µg/assay. A typical agarose gel photo of these treatments is seen in Figure 1.

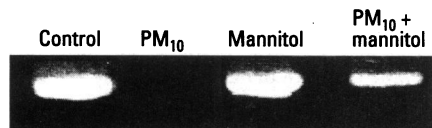


Figure 1. Typical result from a plasmid DNA injury assay. Note depletion of DNA by PM₁₀ and partial protection with mannitol.

Mannitol was added to inhibit hydroxyl radical, at a concentration chosen from dose-response studies as the lowest concentration that caused maximal inhibition.

High-performance Liquid Chromatography Assay. This assay is based on the ability of particle-derived hydroxyl radicals to form the derivative 2,3 dihydroxybenzoic acid (2,3 DHBA) from salicylic acid. One quarter PM₁₀ filter was sonicated in 250 µl of 25 mM salicylic acid in citrate/acetate buffer pH 3.85. The filter was then removed and the salicylic acid/PM₁₀ suspension incubated for 16 hr at 37°C. The suspension was centrifuged to clarity and filtered through a 0.22-µm filter. One hundred microliters of this supernatant was loaded onto a 5 mm Ultrasphere octadecyl silane 250 mm high-performance liquid chromatography (HPLC) column (Alltech, Carnforth, Lancashire, U.K.) and eluted with citrate/acetate buffer. Elution from the column was monitored using an ultraviolet detector, which automatically recorded the elution time of peaks. In control experiments, reagent 2,3 DHBA eluted at 7 min, compared to 15 min for salicylic acid. Using standard concentrations of 2,3 DHBA, we calculated the amount of conversion of salicylic acid to 2,3 DHBA caused by PM₁₀. A blank PM₁₀ filter had no effect in this assay.

Scanning Electron Microscopic Energy Dispersive X-ray Analyses and X-ray Fluorescence Analysis

A PM₁₀ sample was examined on the filter in a Cambridge Stereoscan S90 scanning electron microscope (ISS, Manchester, U.K.), and qualitative analysis carried out via the attached Link energy dispersive X-ray analyzer (EDXA) (Link Analytical Ltd, High Wycombe, U.K.). By focusing the electron beam on the fibers of the filter, we obtained an elemental analysis on the filter matrix material. Focusing on the clumps of particle visible between the filter fibers allowed analysis of the elements in the PM₁₀ particles themselves. The EDXA spectra showed elements from atomic number 11 (sodium) upwards.

X-ray fluorescence analysis was carried out on an untreated PM₁₀ filter using the Rigaku Model 2000 analyzer (Rigaku, Japan), which exposed the entire central 10 mm of the filter to the incident X-ray beam (generated on a rhodium target). The resulting element fluorescence emission from the sample was passed through collimators and diffracted by a lithium fluoride crystal to fall on a scintillation counter,

which scanned through the 2- ϕ range 0 to 99°. Only elements with an atomic number of 9 (fluorine) or greater were recorded.

Statistical Analysis

We used the Minitab statistical analysis program (Minitab, State College, PA) and 1- or 2-way analysis of variance to determine the significance of treatment effects. The Tukey multiple comparison function was used when required.

Results

Free Radical Activity of PM₁₀

Typical output from the DNA plasmid assay is shown in Figure 1, where there is depletion of the supercoiled DNA band by PM₁₀, and protection of the effect by mannitol. When this was quantified by scanning laser densitometry the results were as shown in Figure 2.

In HPLC eluate, a 7-min peak was found in PM₁₀-treated salicylic acid (Figure 3). The standard curve represented 2.1 nmol 2,3 DHBA, which, assuming 1:1 stoichiometry with salicylic acid, means that 2.1 nmol of hydroxyl radical is generated by a quarter filter of PM₁₀.

Effect of Desferrioxamine and Centrifugation on the Free Radical Activity of PM₁₀

When the iron chelator desferrioxamine was added to the plasmid assay, free radical activity was dramatically ameliorated, as shown in Figure 4. Further, when the PM₁₀ was centrifuged to clarity and the clear supernatant tested, plasmid depletion in amounts similar to those of the whole PM₁₀ suspension was noted (in Figure 4). This was again inhibitable by desferrioxamine.

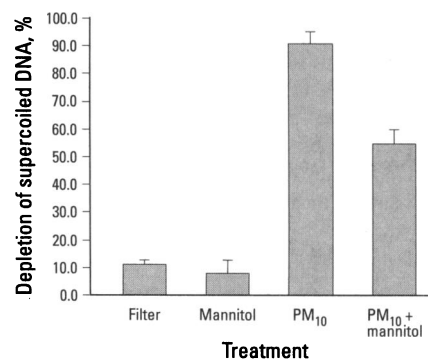


Figure 2. Quantified depletion of supercoiled DNA by blank filter, mannitol, PM₁₀, and PM₁₀+mannitol. Data are shown as mean+SEM and there was a significant protection of the PM₁₀ effect with mannitol ($p < 0.05$).

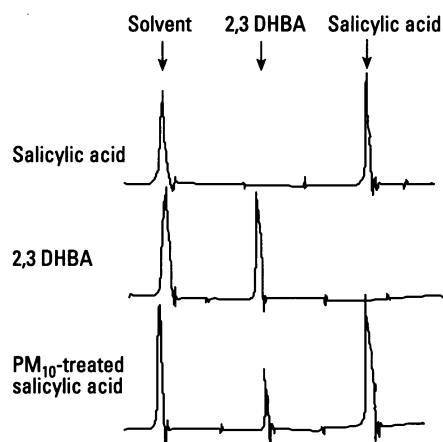


Figure 3. Traces from HPLC elution profiles of untreated salicylic acid, 2,3 DHBA standard, and PM₁₀-treated salicylic acid. Note the appearance of a 2,3 DHBA peak on treatment of salicylic acid with PM₁₀.

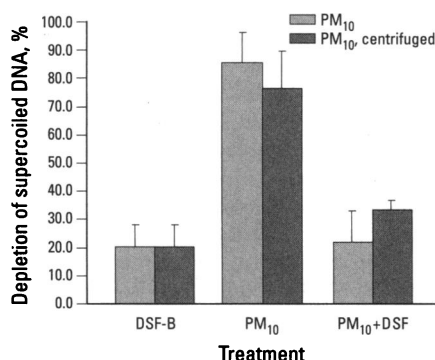


Figure 4. Effects of PM₁₀ and clear supernatant from centrifuged PM₁₀ on plasmid DNA; effect of DSF-B on the DNA depletion. Significant effect of DSF-B compared to PM₁₀ ($p < 0.05$). Data are shown as mean + SEM.

Iron in PM₁₀ Samples

Iron was measured directly on PM₁₀ filters by scanning electron microscopy with an elemental analysis facility, by X-ray fluorescence, and in leachates from PM₁₀, which were obtained using buffer at two pHs.

Scanning electron microscopy of the filter revealed that it was composed of fibers with a distinctive elemental profile characterized by Zn, Ba, Fe, K, Al, and S peaks (Figure 5A). When the beam was focused on particles (avoiding the filter fibers), a very different elemental profile was obtained: no Ba or Zn, diminished K and Ca peaks compared to the filter fibers, and substantial Fe, S, and Cl peaks (Figure 5B). X-ray fluorescence analysis yielded the proportions of major elements: Cl, 21.9%; Fe, 18%; Si, 14.9%, and Na, 10.5%.

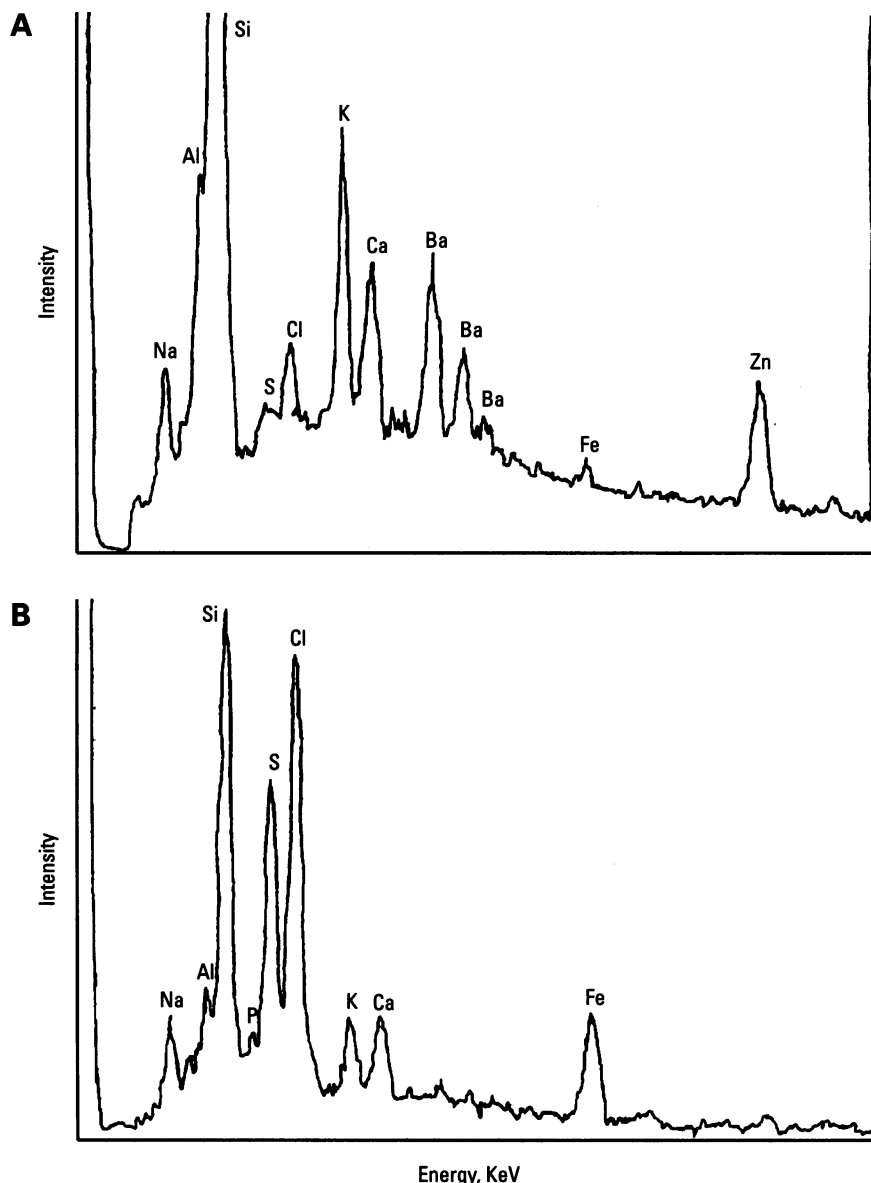


Figure 5. Elemental analysis scan of a PM₁₀ filter. (A) The signal when the beam is focused on the filter fibers; (B) the scan obtained when the beam is focused on particles on the filter.

Figure 6 shows the release of Fe(II) and Fe(III) from PM₁₀ at pH 4.5 and 7.2, respectively, which confirms that large amounts of Fe(III) and small amounts of Fe(II) are released, and that there was more release at acid pH.

Discussion

We recently advanced a unifying hypothesis to explain the pulmonary and extrapulmonary effects of PM₁₀ particles, which was centered around their proposed ability to penetrate the pulmonary interstitium and cause interstitial inflammation (8).

This hypothesis evolved from the original studies of Ferin et al. (13), which demonstrated that ultrafine titanium dioxide was highly inflammogenic and rapidly crossed the epithelium to initiate interstitial inflammation. We demonstrated that ultrafine TiO₂ has free radical activity (14) and hypothesized that this is a factor that enhances interstitial transfer of particles. The relatively huge surface area/unit mass of ultrafine material (7) could be an important factor that leads to oxidative stress to the epithelium and to macrophages. We demonstrated in *in vivo* and *in vitro*

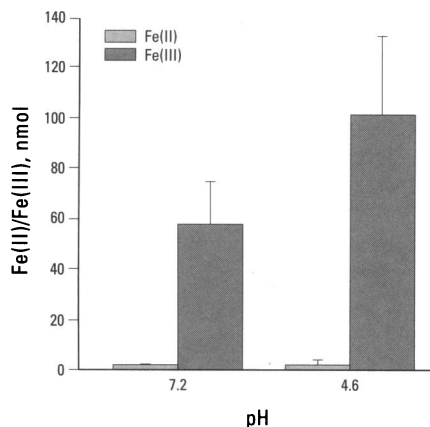


Figure 6. Iron leaching from PM₁₀ at pH 4.5 and 7.2. Data are shown as mean + SEM.

models that oxidative stress leads to loss of epithelial integrity (15,16), which could favor interstitial transfer of particles.

We demonstrate here that PM₁₀ particles have free radical activity as shown by their ability to deplete supercoiled plasmid DNA via OH[•] injury, as confirmed by the protection afforded by mannitol against the PM₁₀ injury. Experiments using desferrioxamine and centrifugation revealed that the OH[•] are generated through reactions that involve Fe, and that this resides either in a soluble or ultrafine fraction.

PM₁₀ particles are thus similar to other pathogenic particles with free radical activity: asbestos (10), glass fibers (10), coal (17), and quartz (18). Iron from asbestos (19,20) and asbestos bodies (21) also has a role in the generation of free radicals. The potentially huge surface area for surface-complexed iron (20), or release of soluble iron presented by an ultrafine fraction of PM₁₀, may cause them to deliver a severe

oxidative stress to cells or lung lining fluid that they encounter.

Not all of the DNA scission was mannitol inhibitable, which demonstrated that other mechanisms for DNA scission are present—not surprising in a heterogeneous mixture such as PM₁₀. PM₁₀ samples released substantial amounts of iron, principally Fe(III), but also trace amounts of Fe(II), which confirms that iron could be released in lung lining fluid and in increased amounts in the macrophage phagolysosome. We assume that there was a reducing source in the PM₁₀ that led to redox cycling with production of Fe(II), which would enhance the capacity to generate OH[•]. The identity of the reducing component or components is unknown.

X-ray fluorescence and analytical scanning electron microscopy revealed a large proportion of Fe in the PM₁₀ sample. The Fe proportion confirmed previous reports that iron, as various salts, is present in SPM samples (22) to the calculated concentration of 0.5 µg/m³ of air (23).

The present data may be of value to explain the association between high PM₁₀ levels and adverse health effects. For instance, the ability of asbestos to stimulate macrophages to release tumor necrosis factor-α can be abolished by chelation of fiber iron (24), which highlights the importance of oxidative stress in particle-mediated cytokine gene transcription in macrophages (25). Asbestos-stimulated transcription of cellular oncogenes *c-fos* and *c-jun* is inhibited by the thiol antioxidant *N*-acetyl-L-cysteine (26) in mesothelial cells. PM₁₀ particles, via the free radical activity demonstrated here, may alter the redox balance of key lung cells and lead to the transcription of proinflammatory cytokines.

The data here contribute a plausible hypothesis for the adverse health effects of PM₁₀ that needs to be further tested. If the iron is present in substantial quantities on the ultrafine fraction, then the relatively huge surface area presented by this fraction could lead to severe oxidative stress in the lung lining fluid and at contact between particles and cells. Our data showed increased leaching of iron at acid pH and suggests that, in the macrophage phagolysosome, low pH could favor release of iron and provide the opportunity for enhanced generation of free radicals. The possibility that PM₁₀ causes exacerbation of airway diseases by an oxidative mechanism, as suggested here, is particularly plausible in view of the authors' own evidence that patients with airway diseases have a measurable deficit in their antioxidant defenses (27). We demonstrated that smokers and patients with exacerbations of COPD and asthma have a systemic lowering of their antioxidant defenses, as measured by a test that detects total antioxidant activity in plasma (27). The deficit in antioxidant defenses shown in plasma is likely to originate from the lungs, in view of the known oxidative stress resulting from the activities of inflammatory leukocytes. These patients are therefore likely to have oxidative stress in the epithelium and lung lining fluid. Further exposure of the oxidatively stressed lung to PM₁₀ particles with free radical activity would represent significant additive oxidative stress that could lead to an aggravation of their condition. The resulting interstitial transfer of particles and pulmonary inflammation could be a significant factor in promoting cardiovascular mortality (8).

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